

1-Deoxymannojirimycin inhibits Golgi-mediated processing of glycoprotein in *Xenopus* oocytes

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We prepared in vitro an mRNA transcript coding for the erythroagglutinating subunit of the kidney bean glycoprotein phytohemagglutinin, E-PHA. The mRNA, injected into *Xenopus* oocytes, synthesized E-PHA carrying two Asn-linked carbohydrate chains, one of which was processed and acquired resistance to endo- β -N-acetylglucosaminidase H, as occurs in the native bean cells. When the mannose analog 1-deoxymannojirimycin, an inhibitor of mammalian Golgi mannosidase I, was included in the oocyte culture medium, the acquisition of endo- β -N-acetylglucosaminidase H resistance was abolished, indicating that also in an amphibian cell the inhibitor blocks a key reaction in Golgi-mediated processing.

1-Deoxymannojirimycin; Oocyte; Glycoprotein processing; Phytohemagglutinin; Golgi complex; (*Xenopus laevis*)

1. INTRODUCTION

Golgi-mannosidase I is the first enzyme acting on the Asn-linked carbohydrate chains of glycoproteins which pass through the Golgi apparatus in mammalian cells. The enzyme removes the α 1,2-linked mannoses from the oligomannose chains producing the Man₅GlcNac₂ structure, a key intermediate for further processing leading to the complex N-linked chains [1]. The mannose analog 1-deoxymannojirimycin (dMM) inhibits mammalian mannosidase I in vivo and therefore blocks the Golgi-mediated processing of Asn-linked chains [2-5]. There appears, however, to be no evidence that such a block can also be obtained in non-mammalian cells. *Xenopus* oocytes are capable of carrying on several steps of processing and transport of foreign proteins synthesized upon mRNA injection [6,7]. We have recently studied expression of the kidney bean lectin phytohemagglutinin (PHA), a well-characterized glycoprotein [8], in *Xenopus* oocytes, and have also shown that the conversion of high oligomannose to complex

carbohydrate chains can take place in these cells [9]. Both in its native cellular environment and in oocytes injected with bean RNA, PHA is synthesized on the endoplasmic reticulum; cotranslationally a signal peptide is removed and two Asn-linked chains are added. During passage through the Golgi complex, the carbohydrate chain distal to the N-terminus is extensively modified and acquires endo- β -N-acetylglucosaminidase H (endo H) resistance [9,10]. Considering the widespread use of *Xenopus* oocytes in studying the molecular cell biology of proteins, we decided to attempt the gaining of more insights into the regulation of carbohydrate chain modification in these cells by examining the effect of dMM on the processing of PHA.

2. MATERIALS AND METHODS

The strategy to obtain polyadenylated mRNA for a PHA polypeptide was as follows. The entire pPVL134 cDNA sequence [11], coding for a lectin-like protein, was excised from the pBR322 vector and inserted into the *Pst*I site of the Bluescript M13-, KS polylinker, vector (Stratagene, San Diego, CA); the fragment was oriented downstream of the T7 promoter and the resulting recombinant plasmid was termed pB7LLP. The coding sequence for the lectin-like protein was excised from pB7LLP by *Sac*I and *Xba*I digestion and sub-

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stituted with a 622 bp fragment containing the coding sequence for E-PHA, isolated from the genomic clone pdlec1 [12] by *Nsi*I and *Xba*I digestion. The resulting plasmid was termed pB7lec1. Note that the *Xba*I site covers the codon for the COOH-terminal leucine both in the lectin-like protein and in the E-PHA sequence and therefore the end of the E-PHA sequence was perfectly reconstructed, in this case followed by the 3'-uncoding region of the lectin-like protein cDNA clone, including a poly(A)tail. Also, at the 5'-end, the 622 bp genomic fragment contained only a TAC trinucleotide preceding the ATG protein initiation sequence of E-PHA.

The method for in vitro transcription of the construct, to yield mRNA which can function in the oocytes, was a modification of the procedure of Krieg and Melton [13]. The 25 μ l in vitro transcription mixture consisted of 1 μ g pB7lec1 linearized with *Eco*RI, 400 μ M rUTP, rCTP and rATP, 40 μ M rGTP, 400 μ M 7mGpppG, 2.5 μ g BSA, 60 U RNasin^T (Promega Biotec, Madison, WI), 30 mM DTT, 8 mM MgCl₂, 50 mM NaCl, 40 mM Tris-Cl (pH 8.0). 10 U T₇ RNA polymerase were then added and the mixture incubated at 37°C. After 30 min the rGTP concentration was adjusted to 400 μ M and again 10 U polymerase added. After an additional 30 min at 37°C, the mixture was brought to 100 μ l with water and adjusted to 10 mM EDTA and 700 mM ammonium acetate; 20 μ g yeast tRNA was then added and the solution (final volume 133 μ l) extracted with phenol-chloroform before precipitation of nucleic acids which was performed by adding 2.5 vols ethanol. After centrifugation and two washings with 80% ethanol, the pellet was resuspended in 25 μ l water and stored at -80°C. This was the solution used for in vitro translation (2 μ l) and oocyte injection (40 nl/oocyte).

Bean (*P. vulgaris* cv. Greensleeves) RNA was prepared from endoplasmic reticulum-bound polysomes isolated from developing cotyledons, as in [14]. In vitro translation was carried out using the reticulocyte lysate (Amersham, Bucks, England) supplemented with 462.5 kBq L-[4,5-³H]leucine (5.62 TBq/mmol, Amersham) per 25 μ l reaction mixture. Oocyte preparation, injection, labelling with [³H]leucine, and homogenization were performed as described [9] but injected oocytes were incubated in Barth medium overnight before 24 h labelling followed by a chase consisting in 6 h medium plus 18 h in medium supplemented with 10 mM unlabelled leucine. In some experiments, oocytes were injected with tunicamycin (40 μ g/ml) and then preincubated for 3 h in Barth medium supplemented with 2.5 μ g/ml tunicamycin before pulse-chase labelling in the presence of the same concentration of the inhibitor. Treatment with dMM was carried out by preincubating oocytes in medium supplemented with 5 mM dMM for 3 h before pulse-chase labelling, which was done in the presence of the same concentration of dMM. Immunoprecipitation using anti-PHA monospecific antibodies, treatment of immunoprecipitated PHA with endo- β -N-acetylglucosaminidase H (from *Streptomyces griseus*; Miles Lab., Elkhart, IN), SDS-PAGE and fluorography were performed as in [9].

3. RESULTS AND DISCUSSION

Since our purpose was to investigate functions performed by oocyte enzymes, it was necessary to avoid the coinjection of undefined bean mRNAs

together with PHA mRNA. We therefore constructed a plasmid, termed pB7lec1, for the in vitro production of a polyadenylated mRNA containing the whole sequence coding for E-PHA, including a 21-amino-acid putative signal peptide. The pB7lec1 transcript, translated in vitro, directed the synthesis of a polypeptide indistinguishable in size from E-PHA produced by a bean RNA preparation (fig.1, cf. lanes 1,2).

Xenopus oocytes were injected with the pB7lec1 transcript or with bean RNA. Oocytes were then labelled with [³H]leucine for 24 h followed by a 24 h chase to allow all processing events to occur, and PHA was immunoprecipitated from the cell homogenates. PHA synthesized by bean RNA gave a broad band (fig.2, lane 1), due to microheterogeneity of the processed carbohydrate chains

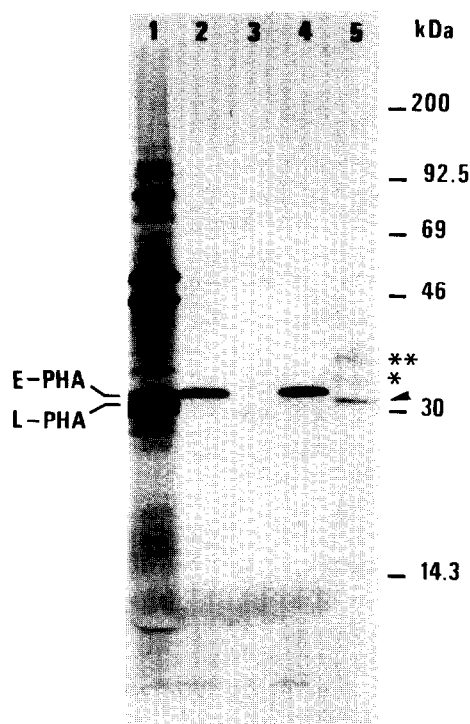


Fig.1. SDS-PAGE and fluorography of E-PHA synthesized in vitro and comparison with E-PHA synthesized in oocytes treated with Tm. Reticulocyte lysate was supplemented with: bean RNA (lane 1), pB7lec1 transcript (lanes 2,4), no RNA (lane 3); total translation products were loaded on the gel. Lane 5: E-PHA isolated from oocytes treated with Tm and injected with pB7lec1 transcript; unglycosylated (arrowhead), singly* and doubly** glycosylated polypeptides are indicated. Positions of molecular mass markers are given on the right.

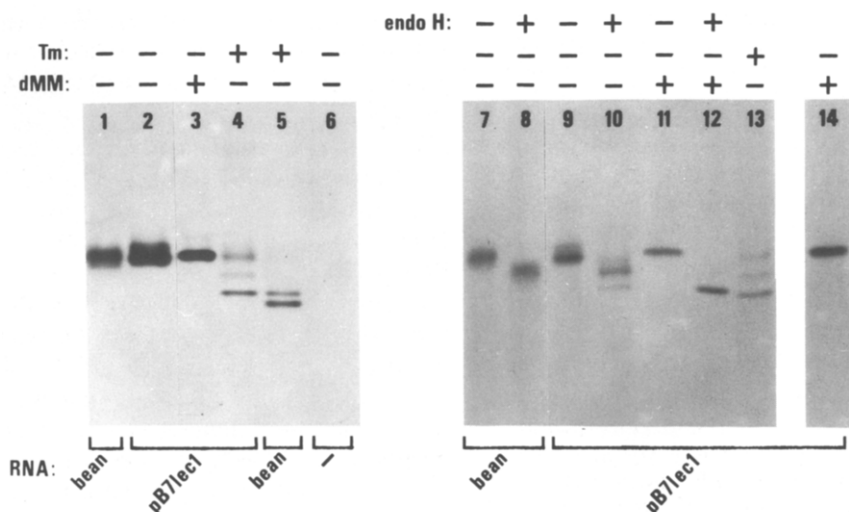


Fig.2. SDS-PAGE and fluorography showing the effect of dMM on the processing of E-PHA. PHA was isolated by immunoprecipitation from oocyte homogenates (lanes 1-14) or a preparation of bean endoplasmic reticulum, which contains PHA in its precursor with only oligomannose carbohydrate chains (lane 15). For endo H digestion, PHA was incubated under the appropriate conditions [10] in the presence of endo H (lanes 8,10,12) or in the absence of the enzyme, as a control (lanes 7,9,11).

of both the E-PHA and L-PHA subunits [9]. The pB7lec1 transcript was efficiently translated in the oocytes (fig.2, lane 2); in this case too the product gave a broad band. These immunoprecipitable proteins were not synthesized in uninjected control oocytes (fig.2, lane 6). Since cloned E-PHA had not been expressed *in vivo* before, we performed experiments to investigate its processing. In the presence of the *N*-glycosylation inhibitor tunicamycin (Tm) the oocytes synthesized unglycosylated E-PHA having electrophoretic mobility slightly higher than that of E-PHA synthesized *in vitro* (fig.1, cf. lanes 4,5; note that inhibition of glycosylation by Tm was not complete in the experiment in lane 5: this allows the detection of singly and doubly glycosylated E-PHA polypeptides as well), and identical to that of E-PHA synthesized in Tm-treated oocytes injected with bean RNA, which is known to undergo correct removal of the signal peptide [9] (fig.2, cf. lanes 4,5; the upper and lower bands in lane 5 represent unglycosylated E-PHA and L-PHA, respectively): these results indicate that the 21-amino-acid sequence at the N-terminus of the cloned E-PHA functions as a removable signal peptide. Endo H cleaves oligomannose chains between the two proximal *N*-acetylglucosamine residues but is unable to cleave complex chains. When E-PHA

synthesized by the pB7lec1 transcript was digested with endo H, most of the material increased in mobility corresponding to removal of one of the two carbohydrate chains (fig.2, lanes 9,10), as occurs in the case of PHA synthesized in oocytes injected with bean RNA (fig.2, lanes 7,8, and see also [9]).

The above results indicated that E-PHA was correctly processed; the effect of dMM on the processing of E-PHA synthesized in oocytes injected with the pB7lec1 transcript was therefore investigated. The inhibitor did not affect the survival of the oocytes and the amount of immunoprecipitable PHA accumulated. E-PHA synthesized in the presence of dMM migrated on SDS-PAGE as a sharp band (fig.2, lane 3), suggesting that its carbohydrate chains were less heterogeneous than those present when the oocytes were not treated with the inhibitor. Endo H digestion (fig.2, lanes 11,12) revealed that in fact dMM fully blocked the formation of complex side chains: both chains could be removed by the enzyme and the product migrated just above unglycosylated E-PHA made in the oocytes in the presence of tunicamycin, as expected for a polypeptide still carrying one inner *N*-acetylglucosamine per glycan attachment site (fig.2, cf. lanes 12,13).

E-PHA made in dMM-treated oocytes was compared with PHA isolated from an endoplasmic reticulum-enriched fraction of developing bean cotyledons pulse labelled with [^3H]leucine: this precursor PHA has not yet reached the Golgi complex and therefore has both side chains still in the oligomannose form [10]. E-PHA made in dMM-treated oocytes migrated slightly below the E-PHA precursor present in the endoplasmic reticulum of bean cells (fig.2, cf. lanes 14,15; upper and lower bands in lane 15 represent E-PHA and L-PHA precursor, respectively): probably, removal of one or a few mannoses also occurred in the oocytes in the presence of dMM. Mammalian cells contain mannosidases insensitive to dMM [1,16]: some of these appear to be endoplasmic reticulum-associated enzymes and can process high mannose chains down to $\text{Man}_6\text{GlcNac}_2$, a structure which is not a substrate for the action of the Golgi glycosyltransferases and mannosidase II which build up the endo H resistant complex chains. Our results would suggest that similar enzymes are also present in the oocytes. Alternatively, some mannose residues could have been removed from E-PHA in a compartment subsequent to the Golgi complex: PHA, unlike other plant storage proteins which are secreted by oocytes [17], is accumulated inside the cells, in an as yet unidentified compartment [9].

The present results indicate that dMM inhibits the conversion of oligomannose to complex oligosaccharides in *Xenopus* oocytes. This suggests that also in an amphibian cell the action of a mannosidase is a prerequisite for the following Golgi-associated processing events.

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